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13. ABSTRACT (Maximum 200 Words) The goal of the proposal studies is to extend our OX-40-specific anti-tumor responses to prostate tumor models by using a protein found on the surface of a T helper subset of leukocytes (OX40). Anti-OX40 delivered into animals with ongoing immune responses are able to clear the tumors and pathogens quicker following the acute immune response and also are left with a greater amount of immunologic "memory". The greater number of memory T cells patients have the recognize these tumors, increases their chance to fight off subsequent metastatic disease. We have found that prostate cancer patients treated with androgen ablation have a large influx of leukocytes into their prostate gland. These cells enter the prostate gland to recognize and destroy tumor cells. Leukocytes that invade the prostate gland after androgen ablation are OX40+. Therefore, we hypothesize that androgen ablation followed by anti-OX40 treatment will enhance anti-tumor immunity in these patients and we propose to exploit our discovery in a prostate cancer mouse model. Fine-tuning our approach to gain preclinical data will help us to understand the most efficient way to augment anti-prostate cancer immunity in prostate cancer patients for future clinical trials.				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	6
References.....	6
Appendices.....	7

INTRODUCTION: OX40 is a cell surface TNF-receptor family member that is primarily found on activated CD4⁺ T cells. Our lab has shown that when this receptor is engaged through its ligand or an anti-OX40 Ab it induces proinflammatory signals that are clinically advantageous when delivered in combination with tumor vaccines or in mice with existing tumors. We also have found expression of OX40 on T cells infiltrating human primary tumors for head and neck cancer, breast cancer, melanoma, and colon cancer. Recently, in collaboration with Dr. Eugene Kwon we have found a large increase of OX40⁺ T cells in the prostate gland of patients that have had androgen ablation twenty days prior to surgery. We now feel there is compelling evidence that OX40 engagement in patients with prostate cancer could have beneficial effects leading to improved clinical results. Therefore, we hypothesize that OX40 engagement in vivo will enhance anti-prostate cancer immunity leading enhanced tumor-free survival. The objective of this study is to obtain enough preclinical data to warrant the design of an OX40-specific clinical trial in patients with prostate cancer. The specific aims of the study are as follows; 1) To determine whether OX40 engagement in vivo will enhance anti-prostate cancer specific immunity, 2) Can OX40 engagement in vivo enhance adoptive immunotherapy against prostate cancer, and 3) To investigate whether the combination of androgen ablation and anti-OX40 treatment are synergistic in the treatment of primary prostate cancer in TRAMP mice. The study design will determine whether OX40-specific tumor immunotherapy treatment regimens will be therapeutic in the TRAMP mouse tumor model. We will look at the efficacy of anti-OX40 therapy in both a tumor transplant setting and in transgenic TRAMP mice destined to succumb to prostate cancer through endogenous tumor formation. The molecular mechanism of anti-OX40 enhanced tumor immunity will also be assessed by gene array analysis of T cells stimulated through OX40. We feel that using anti-OX40 in a prostate cancer setting will ultimately benefit an ongoing anti-prostate cancer immune response leading to enhanced immunity against disease that recurs. This is a therapy that is relatively non-toxic and takes advantage of the body's own defense against malignant cells. The experiments proposed will provide the preclinical data necessary to fine-tune our observations so that we will have a favorable chance to succeed in a prostate cancer clinical in the future.

BODY:

Task #1 as outlined in the approved "statement of work" was to perform dose titration experiments in the s.c. TRAMP tumor model, months 1-12.

We determined through a literature search that the TRAMP-C1 cell line needs to be administered at 0.5×10^6 cells per s.c. injection to get the tumors to take in male BL/6 mice (1, 2). Therefore, we performed a tumor titration in the BL/6 mice starting at 0.5×10^6 and continuing with 1 and 2 million cells. We found that greater than 70% of mice receiving 0.5×10^6 cells formed lethal tumors and 100% of the mice receiving either 1 or 2 million cells formed lethal tumors. Therefore, we performed all of the subsequent "therapy" experiments with 1 or 2 million tumor cells in order to have 100% of the control mice develop tumors. It was noted that the tumors grew quicker in the mice that received 2 million tumors cells compared to mice that had received 1 million tumor cells. The initial anti-OX40-specific therapy experiment inoculated mice with either 1 or 2 million tumor cells and on days 3 and 7 the mice were injected with 250 ug of anti-OX40 or control mice were given rat Ig. Tumor growth was delayed in the anti-OX40 treated groups at both doses of tumor compared to rat Ig treated mice, but eventually the tumor grew out in all the anti-OX40 treated mice. We also tested different doses of anti-OX40 and found that 250 ug gave the best efficacy as far as delayed tumor growth in the TRAMP-C1 model.

Task #3 involved testing anti-OX40 therapy in combination with whole tumor vaccines, 9-24 months.

It has been extensively shown in the recent literature that whole tumor cell vaccines secreting the cytokine, GM-CSF, elicits potent anti-tumor immunity (3). Since anti-OX40 alone was not able to establish tumor-free mice in the experiments performed in Task #1, we obtained a TRAMP-C1 cell line that secretes high amounts of GM-CSF in an attempt to augment anti-OX40-mediated tumor therapy. We attempted experiments in the treatment setting, where the mice were given s.c. TRAMP-C1 and then

treated with anti-OX40 and irradiated TRAMP-C1 secreting GM-CSF. Mice were given s.c. tumor and given anti-OX40 on days 3 and 7 after tumor inoculation and irradiated GM-CSF tumor on days 3, 7 and 10. We found no synergistic or additive when the combination of whole cell vaccine was used in combination with anti-OX40. Future experiments will include injecting the whole tumor cell vaccine and waiting 3 and 7 days prior to administration of anti-OX40. This scenario might allow for the needed "immune priming" period for OX40 to be upregulated on tumor-specific T cells so that anti-OX40 would have a more potent effect. Because of the results obtained in Task #1 we have also tested whether other cytokines administered in vivo will enhance anti-OX40 in the TRAMP-C1 s.c. model. In particular, we obtained a large amount of purified IL-15 from Amgen Inc. and tested combination therapy with anti-OX40. Anti-OX40 was administered on days 3 and 7 after tumor inoculation and IL-15 was given daily from days 3-10. Again, the combination therapy was no better than anti-OX40 alone. Currently, we are testing anti-OX40 in combination with IL-2. The reason driving the IL-2 experiment is that we found that OX40 engagement in vivo upregulates the IL-2 receptor, therefore the combination may have additive or synergistic effects. Future experiments for the s.c. tumor models will include androgen ablation in combination with anti-OX40, which may synergize and augment anti-tumor immunity. We would like to understand which combination therapy works the best prior to moving on to treating TRAMP mice in Task #6.

Task #5 involved gene array analysis of T cells isolated from the TRAMP lung met model, months 24-36.

We have just completed a study that evaluated the genes that were up- or down-regulated in Ag-specific T cells in response to anti-OX40 engagement in vivo in a basic immunology model. This manuscript was recently published in the Journal of Immunology, 2003, 171:5997 and has been added to the appendix. In this manuscript we describe the upregulation of the IL-2 receptor discussed above as well as a number of other important gene differences that will be relevant when performing these studies in the TRAMP model in the future.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Established a TRAMP-C1 tumor cell dose that when injected s.c. is lethal in 100% of mice.
- 2) Established a dose of anti-OX40 that is able to delay tumor growth.
- 3) Determined that the combination of anti-OX40 and GM-CSF secreting tumor vaccine does not enhance therapeutic efficacy at the doses and timing discussed above.
- 4) The combination of anti-OX40 and IL-15 was not synergistic at the doses and timing discussed above.
- 5) Discovered that anti-OX40 engagement in vivo upregulates the IL-2 receptor on Ag-stimulated CD4⁺ T cells.

REPORTABLE OUTCOMES:

Manuscripts:

Prell, R.A., Evans, D.E., Thalhoffer, C., Shi, T., Funatake, C., and **Weinberg, A.D.** (2003) OX40-mediated memory T cell generation is TRAF dependent. Journal of Immunology, 171:5997.

Sugamura, K., Ishii, N., and **Weinberg, A.D.** (2004) Targeting the effector T cell costimulatory molecule OX40 for therapeutic disease intervention. Nature Review Immunology, *In Press*.

Weinberg, A.D., Evans, D.E., Thalhoffer, C., Shi, T., and Prell, R.A. (2004) The generation of T cell memory: a review describing the molecular and cellular events following OX40 (CD134) engagement. Journal of Leukocyte Biology, *In Press*.

Meeting Abstracts:

Invited Speaker:

Weinberg, A.D., Shu, S., and Urba, W. (2004) Engagement of OX40 in vivo enhances anti-tumor immunity. Molecular basis of predictive oncology and intervention strategies, Nice, France. Feb 7-10.

CONCLUSIONS:

During the first year of funding for the prostate cancer work, as it relates to OX40 engagement in vivo, we have become familiar with the TRAMP cell lines and are now comfortable with tumor cell doses and doses of anti-OX40 that show biologic activity in the s.c prostate tumor models. We have found that anti-OX40 treatment of mice with s.c. TRAMP tumors delays tumor growth in approximately 30% of the treated mice. The efficacy of this treatment was not brisk as in other tumor models, therefore we attempted to combine anti-OX40 therapy with other known immune-enhancing strategies. So far the combination therapies have included anti-OX40 with GM-CSF secreting whole tumor cell vaccines as well as the addition of IL-15. We are still working on dosing and schedule issues but as of yet those two treatments were not synergistic or additive in combination with anti-OX40. We have also recently discovered that OX40 engagement dramatically increases the IL-2 receptor on Ag-activated CD4⁺ T cells. IL-2 is now standard therapy for melanoma and renal cancer and will be tested in combination with anti-OX40 in the TRAMP s.c. tumor model. We are currently testing the best combination therapy for anti-OX40 in the s.c. tumor model before we attempt this therapy in the TRAMP mice, which spontaneously develop prostate cancer. After we have completed the cytokine combination studies we will test whether androgen ablation in combination with anti-OX40 will have efficacy in the TRAMP models. Our hope is that anti-OX40 will be a medical product with wide-spread use in the near future. To this end, we have produced a human anti-OX40 Ab that we will be testing in a phase I clinical trial by the fall of this year. The hope is that the knowledge gained from this study will give us the best idea(s) for anti-OX40 use in future phase II and III clinical trials in men with prostate cancer.

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Appendix

OX40-Mediated Memory T Cell Generation is TNF Receptor-Associated Factor 2 Dependent

The Journal of Immunology..... 8

OX40-Mediated Memory T Cell Generation Is TNF Receptor-Associated Factor 2 Dependent¹

Rodney A. Prell,² Dean E. Evans, Colin Thalhofer, Tom Shi, Castle Funatake, and Andrew D. Weinberg³

Tumor necrosis factor receptor-associated factor 2 (TRAF2), an adapter protein that associates with the cytoplasmic tail of OX40, may play a critical role in OX40-mediated signal transduction. To investigate the *in vivo* role of TRAF2 in OX40-mediated generation of Ag-specific memory T cells, we bred OVA-specific TCR transgenic mice to TRAF2 dominant-negative (TRAF2 DN) mice. Following Ag stimulation and OX40 engagement of TRAF2 DN T cells *in vivo*, the number of long-lived OVA-specific T cells and effector T cell function was dramatically reduced when compared with wild-type T cells. We also demonstrate that CTLA-4 is down-regulated following OX40 engagement *in vivo* and the OX40-specific TRAF2 DN defect was partially overcome by CTLA-4 blockade *in vivo*. The data provide evidence that TRAF2 is linked to OX40-mediated memory T cell expansion and survival, and point to the down-regulation of CTLA-4 as a possible control element to enhance early T cell expansion through OX40 signaling. *The Journal of Immunology*, 2003, 171: 5997–6005.

OX40 (CD134), a member of the TNFR superfamily, is expressed on activated CD4⁺ T cells (1). Cross-linking OX40 on the surface of CD4 T cells generates a potent costimulatory signal that enhances T cell proliferation to submitogenic concentrations of Con A, anti-CD3, PHA, and PMA (2–4). Ag-specific T cell responses can also be enhanced by OX40-mediated costimulation (5, 6). OX40 engagement *in vivo* inhibits the peripheral deletion of CD4⁺ T cells that occurs after their expansion in response to superantigens or soluble protein Ag (7, 8), and OX40 knockout mice are deficient in generating normal levels of Ag-specific memory T cells following immunization *in vivo* (7). Thus, OX40 engagement appears to be important for the generation and maintenance of memory T cells following *in vivo* immunization.

TNFR superfamily members coordinate diverse cellular events such as proliferation, differentiation, and programmed cell death (apoptosis) (9–12). The TNFR superfamily can be divided into two subgroups depending on the presence or absence of a cytoplasmic region known as the death domain. Family members with death domains include TNFR-1, Fas/CD95, DR3, DR4, and DR5 (10). This subgroup of receptors possesses a cytoplasmic death domain, which initiates a signaling cascade leading to cell death upon engagement of the receptor (11, 13). In contrast, TNFR family members such as CD27, CD30, CD40, 4-1BB, and OX40 lack a

death domain. They use TNFR-associated factor (TRAF)⁴ adapter proteins that interact with the cytoplasmic tail of TNFR family members and recruit various protein kinases that lead to downstream activation of NF- κ B and c-Jun N-terminal kinase (11, 13). This series of events can ultimately result in cellular proliferation and differentiation (14, 15). Studies have shown that TRAF1, 2, 3, and 5 associate with the cytoplasmic tail of OX40 following receptor cross-linking (16–18). The transcription factor NF- κ B is positively regulated by TRAF2 and TRAF5 and inhibited by TRAF-3 following OX40 ligation (16–18). Although these studies provide a basic understanding of the molecular events that follow OX40 ligation, they were performed by yeast two-hybrid screening or in transiently transfected cell lines. As a result, the physiological relevance of TRAF(s) in OX40-mediated activation of Ag-specific T cells currently remains unclear.

To address the role of TRAF2 in the OX40-mediated generation of memory T cells, we created a double-transgenic mouse by mating TRAF2 dominant-negative (TRAF2 DN) transgenic mice to the OVA-specific TCR transgenic mice (DO11.10). Lymphocytes from the TRAF2 DN transgenic mice express a truncated TRAF2 protein (aa 241–501) in lymphocytes, which contains a deletion of the N-terminal RING and zinc fingers and has been shown to be a DN inhibitor for both TNF and CD40 signaling (19). In this study, we report that functional TRAF2 is essential for optimal expansion and survival of Ag-specific T cells following OX40 engagement *in vivo*. Gene array analysis of Ag-specific T cells stimulated with Ag alone or Ag and anti-OX40 revealed that signaling through OX40 resulted in a 3- to 4-fold decrease in CTLA-4 mRNA and was confirmed by cell surface phenotype analysis. Blocking CTLA-4 during Ag priming *in vivo* was able to partially restore the OX40-specific defect in the TRAF2 DN T cell proliferation early in the response, but could not restore the long-term survival of Ag-specific T cells. These data provide support for a model whereby OX40-enhanced memory T cell development is TRAF2 dependent and the down-modulation of CTLA-4 could be an important early cellular control point for the increase in Ag-specific T cells.

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⁴ Abbreviations used in this paper: TRAF, TNFR-associated factor; DN, dominant negative; DPBS, Dulbecco's PBS; LN, lymph node; WT, wild type.

Materials and Methods

Mice

Four- to six-week-old BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 6–10 wk of age. DO11.10 TCR transgenic mice were obtained from N. Kerkvliet (Oregon State University, Corvallis, OR). TRAF2 DN transgenic mice have been described previously and were obtained from D. Parker (Oregon Health Sciences University, Portland, OR) with the permission of Y. Choi (University of Pennsylvania Medical School, Philadelphia, PA) (19). Both transgenic strains of mice have been backcrossed to BALB/c mice. TRAF2 DN \times DO11.10 were bred at the Earle A. Chiles Research Institute animal care facility (Providence Portland Medical Center) to generate F₁ offspring. All mice were cared for under the National Institutes of Health guidelines.

Phenotyping TRAF2 DN mice

TRAF2 DN \times DO11.10 offspring (>5 wk of age) were tail bled into tubes. The cells were then purified on Lympholyte M (Cedarlane, Hornby, Ontario, Canada) and activated for 48 h on 24-well plates that were precoated with anti-CD3 mAb and anti-CD28. The cells were expanded for 3 days in RPMI-10 containing IL-2 (10 U/ml) and then analyzed for TRAF2 expression by Western blot analysis. An anti-TRAF2 Ab (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect TRAF2 protein expression by Western blot analysis.

Adoptive transfer and immunization

The protocol for adoptive transfer of wild-type (WT) DO11.10 or TRAF2 DN⁺ DO11.10 T cells was a modification of that previously described (20). Splenic B cells were depleted by nylon wool fractionation, as previously described (21). A total of $1\text{--}2 \times 10^6$ TRAF2 DN KJ1-26⁺ or WT KJ1-26⁺ T cells in Dulbecco's PBS (DPBS) was adoptively transferred i.v. into the tail vein of age- and sex-matched BALB/c mice. Adoptively transferred mice were rested for 2 days and then immunized by s.c. injection in the back with 0.5 mg of OVA plus 50 μ g of rat IgG or 50 μ g of anti-OX40 in 0.2 ml of DPBS also injected s.c. (day 0). The following day (day 1), the mice received a second s.c. injection of rat IgG (50 μ g) or anti-OX40 (50 μ g).

Abs and reagents

CyChrome anti-CD4 was purchased from BD PharMingen (La Jolla, CA). Biotinylated KJ1-26 was kindly provided by N. Kerkvliet (Oregon State University, Corvallis, OR). PE-Streptavidin was purchased from Jackson ImmunoResearch (West Grove, PA). Purified and PE-conjugated anti-CTLA-4 (UC10-4F10-11) were purchased from BD PharMingen. The hybridoma that produces the rat anti-murine OX40 mAb (OX86) was obtained from the European Cell Culture Collection. Rat IgG (Sigma-Aldrich, St. Louis, MO) was used as a control for all in vivo injections of OX86. The OX40L:lg fusion protein (provided by Xenova Pharmaceuticals, Cambridge, U.K.) was used to stain for OX40 expression on activated T cells. OVA (Sigma-Aldrich) was diluted in DPBS and filter sterilized through a .22- μ m filter.

Proliferation assay

The indicated numbers of KJ1-26⁺ T cells were stimulated for 72 h in 96-well flat-bottom plates. The plates were pulsed with 1 μ Ci of [³H]thymidine during the last 12 h of culture. The cells were harvested on a Tomtec 96-well plate harvester, and thymidine incorporation was counted on a Trilux 1450 microbeta liquid scintillation counter (Wallac, Gaithersburg, MD).

Flow cytometric analysis

Approximately 2×10^6 cells were washed and resuspended in FACS buffer (1% FBS, 0.1% sodium azide in PBS). Cells were labeled with Cy-CD4 and biotinylated KJ1-26, followed by PE-labeled streptavidin. A minimum of 100,000 events was collected by listmode acquisition on a FACScan (BD Biosciences, San Jose, CA) and analyzed using CellQuest software.

Staining for CTLA-4. Draining lymph nodes (LNs) were harvested 3 days postimmunization and were stained in RPMI with 10% FCS at 37°C with anti-CTLA-4-PE (UC10-4F10-11 clone (BD PharMingen)) for 3 h. The cells were then washed and stained with Cy-CD4 and biotinylated KJ1-26, followed by FITC-labeled streptavidin. Upon analysis, cells were gated on KJ1-26⁺/CD4⁺ cells and analyzed for CTLA-4; histograms are shown vs an isotype control in Fig. 1A. The difference in geometric mean for an isotype control-PE Ab vs anti-CTLA-4-PE is represented by bar graphs in Fig. 1B (three mice/group).

Cytokine detection

LN cells were incubated, under the indicated conditions, for 24 h in a 24-well flat-bottom plate at 2.5×10^6 cells/ml in RPMI containing 10% FBS. Cytokines were detected by a standard sandwich ELISA (BD PharMingen).

Gene array analysis

Lateral axillary LNs were collected 3 days after immunization and processed into a single cell suspension. The cells were stained for 20 min on ice with biotinylated KJ1-26 (0.5 μ g/ 10^6 cells). Cells were washed and incubated with anti-biotin beads (Miltenyi Biotec, Auburn, CA) for 20 min on ice. The KJ1-26⁺ T cells were purified by magnetic cell sorting using the Automacs (Miltenyi Biotec). The cells were run over the column, and the positive fraction was collected to obtain cells enriched for KJ1-26⁺ cells. The positive fraction was then rerun over the column to enrich to high purity. Total RNA from the KJ1-26⁺ cells was collected using RNeasy (Qiagen, Valencia, CA), according to the manufacturer's instructions. The RNA samples were then sent to the Oregon Cancer Center Affymetrix microarray core facility (Beaverton, OR) for further analysis (www.ohsu.edu/gmar/). The core facility produced cRNA from the samples and probed the Affymetrix MG-U74Av chip, which includes 12,000 genes. The probed arrays were scanned and then analyzed by the Affymetrix software MAS4.0. Table II shows representative genes that were consistently increased or decreased upon OX40 engagement in three separate biologic replicate samples.

Statistical analysis

Comparison of the means was made using the least significant difference multiple comparison *t* test or Dunnett's *t* test for pairwise comparisons (Statview statistical software; SAS Institute, Cary, NC). Values of $p \leq 0.05$ were considered statistically significant. Microarray data analysis and statistics were performed in the Affymetrix Bioinformatics & Biostatistics Core of the Oregon Health Science University (Portland, OR) Gene Microarray Shared Resource.

Results

Expression of endogenous TRAF2 and transgenic TRAF2 DN protein in activated PBLs

To examine whether TRAF2 is important for mediating the downstream signaling through OX40 on Ag-specific CD4⁺ T cells, we created a novel double-transgenic mouse by breeding TRAF2 DN transgenic heterozygous mice with homozygous DO11.10 TCR transgenic mice. The mice were phenotyped for expression of the TRAF2 protein expression by Western blot analysis of in vitro activated PBL. Both the endogenous TRAF2 and the TRAF2 DN protein were detected using this procedure (Fig. 1). Endogenous TRAF2 was undetectable in naive PBLs (data not shown). However, in vitro stimulation and expansion of PBL allowed for the detection of the WT TRAF2 protein as a 50-kDa band and the transgenic TRAF2 DN protein (*lanes 3 and 4*) as a 31-kDa band (19). We also observed that expression of the TRAF2 DN transgene had a negative effect on WT TRAF2 protein detection following activation with anti-CD3, anti-CD28, and IL-2. Endogenous WT TRAF2 protein detection was approximately 20-fold greater in WT lymphocytes (*lanes 1 and 2*) compared with lymphocytes isolated from TRAF2 DN T cells (*lanes 3 and 4*).

When the TRAF2 DN/DO11.10 T cells were compared with WT DO11.10 T cells for Ag-specific T cell function, we found no inherent defects in proliferation or cytokine production. The only difference we observed was a 2-fold increase in IL-4 production by naive TRAF2 DN T cells stimulated with Ag, which is consistent with a recently published report (22). Most importantly, we needed to establish whether OX40 expression was similar in TRAF2 DN and WT T cells stimulated with Ag. Potentially, a defect in TRAF2 signaling could result in a failure to up-regulate OX40. OX40 was induced to a similar degree on both WT and TRAF2 DN DO11.10 T cells after stimulation with OVA in vitro for 24 or 48 h (Fig. 1B shows 48-h time point). Because TRAF2 DN and WT T cells

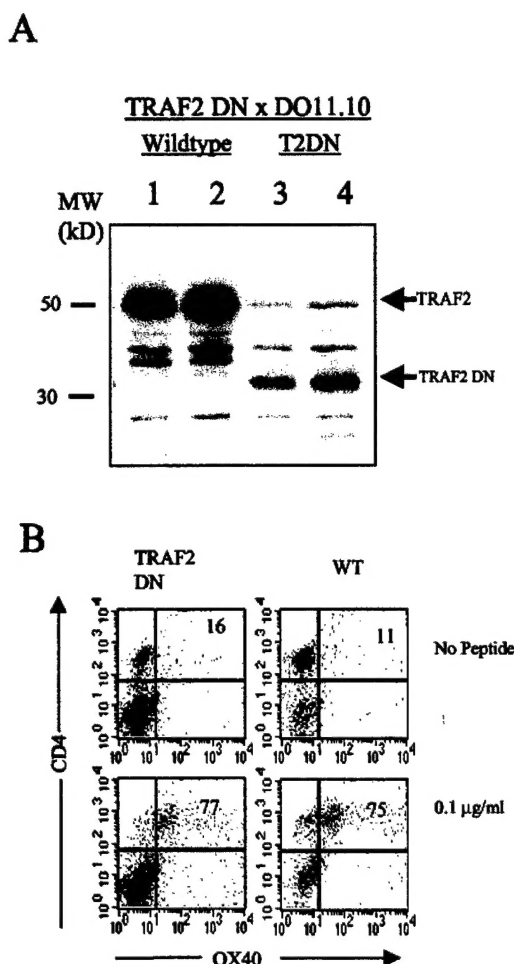


FIGURE 1. Detection of endogenous TRAF2 and TRAF2 DN protein expression in double-transgenic mice and OX40 expression from WT and TRAF2 DN T cells. **A**, Western blot analysis of in vitro activated PBL isolated from TRAF2 DN transgenic (TRAF2 DN) and nontransgenic (WT) littermates. Cell lysates (1×10^6 cell equivalents) were extracted from anti-CD3- and anti-CD28-stimulated PBL, and were probed using an anti-TRAF2 polyclonal Ab that recognizes a carboxyl-terminal epitope. Endogenous TRAF2 (50 kDa) and TRAF2 DN (31 kDa) proteins are indicated by arrows. **B**, OX40 expression on T cells isolated from WT or TRAF2 DN mice stimulated with OVA₃₂₃₋₃₃₉ for 48 h. Cells from each group were normalized to 1.2×10^5 CD4⁺KJ1-26⁺/well and stimulated with the OVA₃₂₃₋₃₃₉ peptide (0.1 μ g/ml). Cells were stained with Cy-CD4, biotinylated KJ1-26/PE-streptavidin, and muOX40L:Ig/ α HuFITC. The numbers in the upper right quadrant represent the percentage of OX40⁺ of the total CD4⁺ cells.

responded similarly when stimulated with Ag in vitro, any differences in how these two groups respond to Ag in combination with an agonist Ab to OX40 in vivo should be due to events that occur downstream to OX40 signaling.

Functional TRAF2 is required for an optimal response to OX40 engagement in vivo

To examine the role of TRAF2 in OX40-mediated expansion and survival of OVA-specific CD4⁺ T cells in vivo, T cells from DO11.10 WT or TRAF2 DN \times DO11.10 double-transgenic mice were transferred into naive BALB/c recipients. Following adoptive transfer, the mice were immunized with OVA and treated with an agonist Ab to OX40 or a control Ab (rat Ig). The effect of OX40 engagement on expansion of OVA-specific T cells was monitored by staining PBLs with a CD4 Ab and a KJ1-26 Ab (an Ab that

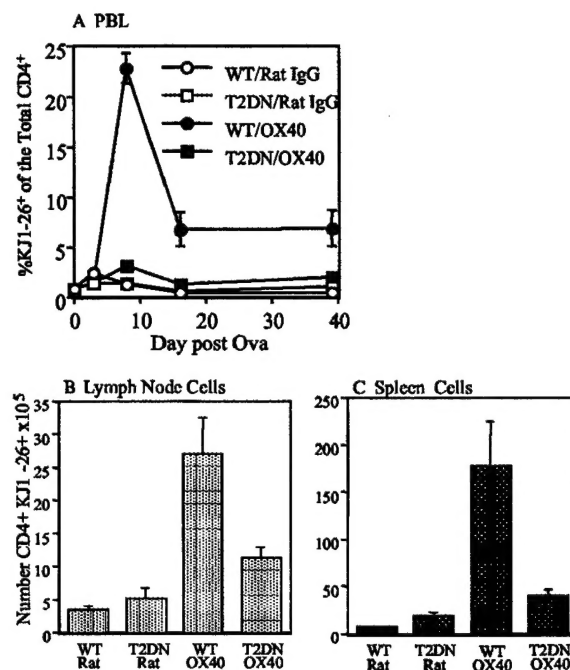


FIGURE 2. TRAF2 DN protein expression inhibits the generation/survival of Ag-specific T cells after OX40 engagement in vivo. A total of 1×10^6 TRAF2 DN⁺ KJ1-26⁺ (TRAF2 DN; squares) or littermate control (WT; circles) KJ1-26⁺ T cells was transferred into BALB/c mice. Two days later, mice were primed with OVA and rat IgG (open symbols) or OVA and anti-OX40 (filled symbols), as described in *Materials and Methods*. On the indicated days, mice were tail bled and PBL were isolated and stained with anti-CD4-Cy and biotinylated KJ1-26 mAb, followed by streptavidin-PE, and analyzed by flow cytometry. Unprimed mice were used as day 0 controls. On day 39, the total number of CD4⁺ KJ1-26⁺ T cells was calculated in the draining LNs (**B**) and spleen (**C**). Numbers represent mean \pm SE of five mice/group.

recognizes the DO11.10 TCR clonotype) (23). As previously observed (23), there was a large increase of Ag-specific T cells in the peripheral blood 7 days after immunization and anti-OX40 treatment compared with the rat Ig-treated WT group (23 vs 2% of CD4⁺ T cells) (Fig. 2A). However, when the TRAF2 DN group was immunized and treated with anti-OX40, there was only a modest increase in the Ag-specific T cells compared with the rat Ig-treated group (3 vs 2% of CD4⁺ T cells). Although the differences in the OX40-treated WT and TRAF2 DN groups were dramatically different throughout the time course, there was no difference observed in the rat Ig-treated groups. Similar trends were observed in the spleen and the draining LNs 30 days after immunization (see Fig. 2, B and C).

We next examined the effects of TRAF2 DN transgene expression on OX40-mediated generation of long-term T cell memory. The experimental groups were designed exactly as in Fig. 2; however, they were sacrificed 196 days after immunization (Table I). OX40 engagement increased the number of WT KJ1-26⁺ T cells by 10-fold in the spleen and peripheral blood, and 5-fold in the LNs, but did not enhance survival of KJ1-26⁺ T cells that expressed the DN form of the TRAF2 protein.

TRAF2 DN T cells stimulated with OVA and anti-OX40 in vivo respond less vigorously upon re-exposure to Ag ex vivo

Up to now, we have examined the importance of TRAF2 in OX40-enhanced T cell expansion and survival in vivo. Next we assessed whether TRAF2 DN day 3 effector T cells exposed to Ag and anti-OX40 in vivo retained their capacity to produce IL-2 and

Table I. TRAF-2 is required for long-term survival of OVA-specific T cells following OX40 engagement (196 days post OVA immunization)

Treatment ^a	Number CD4 ⁺ KJ1-26 ⁺ cells ($\times 10^{-4}$)		Percentage of CD4 ⁺ KJ1-26 ⁺
	Spleen	LN	
WT/Rat IgG	11.6 \pm 1.8	0.9 \pm 0.2	0.4 \pm 0.1
TRAF2 DN/ Rat IgG	10.5 \pm 2.3	0.5 \pm 0.1	0.3 \pm 0.1
WT/OX40	118.6 \pm 28.6 ^b	5.1 \pm 1.6 ^c	4.4 \pm 1.0 ^b
TRAF2 DN/ OX40	14.4 \pm 1.9	1.1 \pm 0.2	0.3 \pm 0.1

^a 1×10^6 TRAF-2DN⁺KJ1-26⁺ or WT KJ1-26⁺ T were adoptively transferred (i.v.) into age and sex matched BALB/c recipient mice. Adoptively transferred mice were rested for 2 days and then immunized (s.c.) with 0.5 mg of OVA. Mice were then injected i.v. with 50 μ g of rat IgG or 50 μ g of anti-OX40 (day 0). The following day (day 1) mice received a second injection of rat IgG (50 μ g) or anti-OX40 (50 μ g). On day 196 after OVA injection, cells were counted, stained with anti-CD4 and KJ1-26 mAb, and analyzed by flow cytometry as described in *Materials and Methods*. The data represent mean \pm SEM from five mice per treatment group.

^b Significant difference from all other treatment groups ($p < 0.008$ Dunnett's *t* test).

^c Significant differences from all other treatment groups ($p < 0.05$ Dunnett's *t* test).

IFN- γ upon re-exposure to Ag directly ex vivo. Immunization of recipients receiving WT KJ1-26⁺ T cells stimulated with OVA and anti-OX40 resulted in a substantial increase in IL-2 (30-fold) and IFN- γ (27-fold) production compared with WT recipients immunized with OVA alone (Fig. 3, C and D). Anti-OX40 also augmented IL-2 and IFN- γ production from TRAF2 DN⁺ KJ1-26⁺ T cells. However, the relative increase was lower than that observed in the WT mice treated with anti-OX40. The WT T cells stimulated with Ag and anti-OX40 produced approximately 4-fold more IL-2 and IFN- γ when compared with TRAF2 DN T cells stimulated with Ag and anti-OX40 (Fig. 3, C and D). The ex vivo proliferative response was also 2.5-fold greater in WT T cells compared with T2DN T cells that were exposed to Ag and anti-OX40 (Fig. 3B). The data indicate that OX40 engagement in vivo produces effector T cells that are more responsive to Ag, and expression of functional TRAF2 is critical in the generation of these efficient effector T cells.

TRAF2 DN protein expression inhibits OX40-mediated in vivo expansion of Ag-specific T cells within the draining LNs

The data shown in Fig. 3 suggest that anti-OX40 exerted its effects early on Ag-specific T cells (by day 3 after immunization) within the draining LNs. We next investigated the impact of TRAF2 DN transgene expression on early T cell expansion and cell size after Ag stimulation and OX40 engagement. Fig. 4A shows that the relative percentage of Ag-specific T cells was similar in all three groups on day 2 after immunization, but there was a small increase in both the T2DN and WT group stimulated with anti-OX40 on day 3. A large increase in the percentage of Ag-specific WT T cells stimulated with anti-OX40 occurred between days 3 and 4 (from 14 to 36% of the CD4⁺ T cells), and this increase was not observed in the TRAF2 DN group stimulated with anti-OX40 nor the WT rat Ig control group. Fig. 4B represents the cell size of Ag-specific T cells (gated on day 3 stimulated OVA-specific T cells) in the three groups depicted in Fig. 4A. T cells from both the WT and T2DN groups stimulated with anti-OX40 in vivo show an increase in cell size and appear more blastlike than the WT T cells immunized with Ag alone. The data indicate that there is a large expansion of Ag-specific T cells that occurs within the day 3–4 timeframe that is OX40 mediated and TRAF2 dependent; however, the OX40-mediated increase in T cell size appears to be independent of TRAF2 signaling.

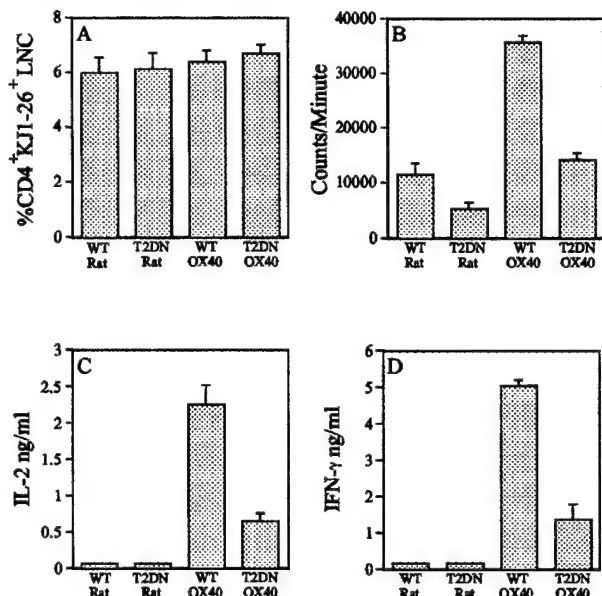


FIGURE 3. Expression of TRAF2 DN protein inhibits OX40-enhanced effector T cell response to Ag. Normal BALB/c mice received 1×10^6 TRAF2 DN⁺ KJ1-26⁺ (TRAF2 DN) or littermate control (WT) KJ1-26⁺ T cells. Two days later, mice were primed with OVA and given either rat IgG or anti-OX40, as described in *Materials and Methods*. Three days after OVA injection, LN (popliteal, axillary, and lateral axillary) were removed and CD4⁺ lymphocytes were stained for the percentage of KJ1-26⁺ within the CD4⁺ T cell compartment (A), or restimulated ex vivo with OVA_{323–339} (1 μ g/ml) peptide to measure proliferation (B), IL-2 production (C), and IFN- γ production (D). Because all the groups had a similar percentage of KJ1-26⁺ T cells (A), the cultures for B, C, and D were normalized for KJ1-26⁺ cells by adding the same number of LN cells. No proliferation or cytokine production was detected in cultures stimulated with an irrelevant peptide (data not shown). Numbers represent mean \pm SE of four to five mice per treatment group.

Gene array analysis of RNA isolated from Ag-specific LN T cells stimulated with or without anti-OX40

We observed several differences in Ag-specific T cell function 3 days after OX40 engagement in vivo (see Figs. 3 and 4). Therefore, we set out to elucidate what genes were increased or decreased upon OX40 engagement within the Ag-specific T cell population 3 days after immunization, by microarray analysis. Purity of the Ag-specific T cell population and swift cell-sorting capabilities were the two greatest concerns for the microarray analysis. We found that magnetic separation of the Ag-specific T cell populations (by AutoMacs cell sorter via the KJ1-26 Ab) was the fastest, most efficient way to obtain high yields of pure cells leading to intact RNA. The KJ1-26⁺ T cells in the postsort LN samples obtained from 3 day immunized mice with or without anti-OX40 stimulation were >92% pure for all samples subjected to microarray analysis (data not shown). RNA was isolated directly ex vivo from the postsort samples, labeled, and hybridized to an Affymetrix murine chip (MG-U74Av2). This particular mouse array contains the majority of known mouse gene products and some mouse expressed sequence tag sequences that have not been assigned to a specific protein (~12,000 genes in total). Of the 12,000 genes screened, 7,983 were expressed by the day 3 activated OVA-specific T cells. Table II shows representative known genes whose expression was consistently increased or decreased in Ag-specific T cells upon anti-OX40 administration in vivo in three separate biologic replicate experiments. In the three replicate experiments, 44 genes were consistently increased and 112 genes were decreased greater than 2-fold upon OX40 engagement in vivo. Table II represents a

Table II. Genes that were increased or decreased 2-fold or greater, anti-OX40 vs rat Ig^a

<i>p</i> Value ^b	Average Fold Difference ^c	Genes
	Increased	
0.003	24.6	Z72486 ^d : polymerase (DNA directed), δ 2, regulatory subunit (50 kDa)
0.0020	4.4	M26271: mouse interleukin 2 receptor gene
0.036	4.3	U43918: proliferation-associated protein 1 mRNA
	Decreased	
0.003	-69.5	U89491: microsomal epoxide hydrolase (Eph1)
0.006	-13.5	U32395: Max-interacting transcriptional repressor (Mad4) mRNA
0.026	-5.7	U00937: GADD45 protein (gadd45)
0.018	-5.5	AJ250489: Ramp1 gene
0.005	-4.8	Z16410: btg1
0.042	-4.5	AB016589: IKK-I mRNA for inducible I κ B kinase
0.014	-4.3	Z54283: B-cell-specific coactivator BOB.1/OBF.1
0.0107	-4.0	L24495: CD27
0.021	-4.0	U18372: CD37 Ag
0.042	-4.0	AF056187: insulin-like growth factor 1 receptor
0.017	-3.9	M83649: Fas Ag
0.035	-3.5	AF099972: schlafen 1 (Slfn1)
0.037	-3.2	X05719: CTLA-4
0.020	-3.0	M36058: mouse lymph node homing receptor (L-selectin)
0.004	-2.9	X62646: IL-6 signal transducer (gp130)
0.005	-2.8	M64292: B-cell translocation gene 2, anti-proliferative
0.021	-2.5	U95826: cyclin G2 mRNA

^a This table represents "known" genes that were up- or down-regulated of the 156 genes that were found to be different on day 3 after immunization.

^b Values of *p* were determined by a standard *t* test evaluation. Genes that showed OX40-specific differences in their *p* values >0.05 were omitted from Table II.

^c The difference between RNA isolated from stimulated with Ag and OX40 vs Ag and rat Ig groups as calculated by the Affymetrix MAS4.0 software. These numbers represent the mean of three separate biologic replicate experiments.

^d These numbers represent the accession number for which information about these genes can be found in the public database such as the National Library of Medicine at www.ncbi.nlm.gov/entrez/query.fcgi.

comparison of gene transcript expression levels in which statistically significant differences were found by a standard two-sample *t* test comparison.

Because the TRAF2 DN T cells are not as responsive as WT T cells to OX40 stimulation *in vivo*, we were interested in determining which genes might be modulated by decreased TRAF2 signaling in response to OX40 engagement. Another gene array experiment similar to the one described above (day 3 time point) was performed comparing RNA isolated from TRAF2 DN or WT D011.10 T cells stimulated with OVA and anti-OX40. Of the genes that were either up-regulated or down-regulated in T cells upon OX40 engagement in WT T cells (see Table II), expression of only 20 were altered in the TRAF2 DN T cells stimulated with anti-OX40. CTLA-4 and L-selectin were two immune-specific gene products whose mRNA levels were decreased upon OX40 engagement in WT T cells, and this OX40-specific decrease was not observed in RNA isolated from the anti-OX40-stimulated TRAF2 DN T cells. For this study, we focused on the decrease observed in CTLA-4 mRNA expression because of its known capacity to down-regulate T cell function during Ag-specific activation (24). Table III depicts CTLA-4 mRNA expression in four separate experiments (three of which were collated from Table II), and two of the comparisons included a TRAF2 DN group stimulated with anti-OX40. The absolute numbers in Table III represent the raw numbers obtained from Affymetrix software MAS 4.0 used to analyze the probed array. As shown in Table III, CTLA-4 mRNA levels in TRAF2 DN T cells stimulated with Ag and anti-OX40 were partly to completely restored to the levels observed with Ag and rat Ig. Therefore, the data suggest that OX40-mediated down-regulation of CTLA-4 may be a TRAF2-dependent event and could be an integral part of the OX40 stimulatory pathway.

CTLA-4 expression and function in OX40-stimulated T cells

To further support the gene array data, draining LNs were harvested 3 days after immunization with or without anti-OX40 treatment, and the Ag-specific WT T cells were stained for CTLA-4 surface expression. As a negative control, T cells from unimmunized mice that had received OVA-specific T cells were stained for CTLA-4 surface expression. Fig. 1A shows that there was virtually no expression of CTLA-4 on Ag-specific T cells isolated from unimmunized mice. There was a lower level of CTLA-4 expression on Ag-specific T cells obtained from mice stimulated with OVA and anti-OX40 when compared with the OVA and rat Ig control group (Fig. 5A). We next compared CTLA-4 staining on *ex vivo* isolated Ag-activated T cells for both anti-OX40 and rat Ig groups in WT or TRAF2 DN T cells. To obtain statistical confidence, we represented the groups as the average fold-change in the geometric means for CTLA-4 (means were obtained from three mice/group). We found a statistically significant decrease in CTLA-4 surface expression in the Ag-specific WT T cells upon OX40 engagement, but not in the TRAF2 DN groups (Fig. 5B). Thus, the staining data confirmed the gene array data and suggest that OX40 engagement may enhance T cell activation and proliferation by decreasing surface cell expression of CTLA-4.

We hypothesized that a short circuit in OX40 signaling via the aberrant TRAF2 adapter molecule could lead to increased B7-CTLA-4 interaction and decreased T cell function. Therefore, to examine the biologic significance of the OX40-specific decrease in CTLA-4 expression, we determined whether blocking CTLA-4 could reverse the OX40-specific TRAF2 DN T cell defect. In Fig. 6A, mice were immunized either with OVA, OVA and anti-OX40, OVA and anti-CTLA-4 (blocking Ab), or OVA and anti-OX40 and

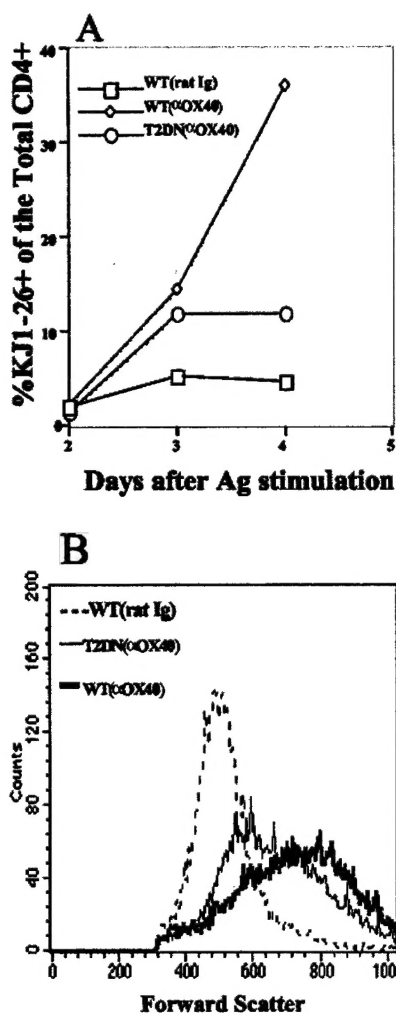


FIGURE 4. TRAF2 DN protein expression inhibits OX40-mediated expansion of Ag-specific T cells within the draining LNs, but does not inhibit the increase in cell size. Normal BALB/c mice received 1×10^6 TRAF2 DN⁺ KJ1-26⁺ (TRAF2 DN) or littermate control (WT) KJ1-26⁺ T cells. Two days later, mice were primed with OVA and given either rat IgG or anti-OX40, as described in *Materials and Methods*. **A**, Cells were isolated from either WT or TRAF2 DN groups stimulated with or without anti-OX40. The T cells from this time course experiment were pooled from the draining LNs of five to seven mice/group for gene array analysis and assessed in this figure for percentage of KJ1-26⁺ T cells of the total CD4⁺ T cells. **B**, The forward scatter plot is derived from LN T cells from the day 3 time point in **A**. Cells were stained, as described in *Materials and Methods*, and gated on KJ1-26⁺ T cells. The plot represents the forward scatter of the KJ1-26⁺ T cells from WT groups stimulated with Ag and rat Ig or Ag and anti-OX40 and TRAF2 DN groups stimulated with Ag and anti-OX40.

anti-CTLA-4. The mice were injected with two doses of 100 μ g of anti-CTLA-4, which was a dose previously shown to enhance Ag-specific CD4⁺ T cell expansion in this same model system (23). Anti-CTLA-4 alone increased the percentage of KJ1-26⁺ T cells early in the blood on day 4 in both WT and TRAF2 DN groups. The combination of anti-CTLA-4 and anti-OX40 in vivo was no better than anti-OX40 alone in stimulating WT T cells. In contrast, the percentage of TRAF2 DN KJ1-26⁺ T cells detected in the blood on day 4 was highest when mice received the combination of anti-OX40 and anti-CTLA-4 compared with anti-OX40 alone or anti-CTLA-4 alone. However, the combined treatment was short-lived, and by day 29 the percentage of Ag-specific T cells in the TRAF2 DN group had returned to background levels. A similar

Table III. Day 3 Postimmunization CTLA-4 gene array analysis

Experiment No. ^a	Absolute Number for CTLA-4 Expression ^b			Fold Difference ^c
	WT Ag	WT Ag OX40	TRAF2 DN Ag OX40	
1	465.7	96.1	209.6	4.4
2	235.4	81.0	ND ^d	2.9
3	234.2	101.7	ND ^d	2.3
4	208.0	78.5	208.4	3.7

^a Each experiment represents a separate RNA sample that was obtained in an independent experiment run on separate days.

^b The absolute numbers were obtained from the Affymetrix software MAS4.0 for CTLA-4 expression and represent a relative value (not an exact microgram amount).

^c The fold difference was calculated from the Affymetrix MAS4.0 software and represent the difference in CTLA-4 RNA produced by WT Ag vs WT AgOX40.

^d ND, Not done.

rapid decline in the percentage of peripheral blood KJ1-26⁺ T cells was observed in both WT and TRAF2 DN T cells after immunization with Ag and anti-CTLA-4 alone. Next, the number of Ag-specific T cells in the draining LNs was assessed after the various treatments described above (Fig. 6B). The mice analyzed in Fig. 6B received half the dose of anti-CTLA-4 (50 μ g/injection) (23). At 50 μ g/injection, anti-CTLA-4 alone does not have immune enhancing properties in vivo in this model (unpublished observation, D. Evans and A. Weinberg). This strategy was used to test whether the OX40-specific TRAF2 DN phenotype could be reversed without observing a T cell-enhancing effect in mice injected with anti-CTLA-4 alone. Similar to what was observed in the blood as well as in Fig. 5A, anti-OX40 alone dramatically enhanced expansion of WT, but not TRAF2 DN Ag-specific T cells in the LNs. The lower dose of anti-CTLA-4 alone was ineffective at enhancing WT or TRAF2 DN T cell expansion, but the combination of anti-OX40 and anti-CTLA-4 resulted in a synergistic increase in the number of TRAF2 DN KJ1-26⁺ T cells on day 4 after Ag stimulation. Again, no increase in WT Ag-specific T cell expansion was observed when the combination of Abs was injected vs anti-OX40 alone. The combination of anti-OX40 and anti-CTLA-4 (50 μ g/injection) was less effective in enhancing LN expansion of TRAF2 DN T cells compared with WT T cells. Thus, blocking CTLA-4 partially overcame the TRAF2 DN early proliferative defect in OX40-specific signaling within Ag-specific T cells, and may be a control point for OX40-enhanced early proliferation. But other, as of yet unidentified, TRAF2-dependent signals/gene transcripts are most likely responsible for the OX40-mediated increase in long-term memory T cell survival.

Discussion

We bred a novel double-transgenic mouse (TRAF2 DN \times DO11.10) to examine the effects of disrupting TRAF2 signaling within Ag-specific T cells following OX40 engagement in vivo. Our results demonstrate that the generation of Ag-specific memory T cells following OX40 engagement is severely impaired in T cells that lack normal TRAF2 function. The data show that there are two distinct phases of OX40-mediated enhancement of Ag-specific CD4⁺ T cell memory generation. The first phase is the initial expansion of Ag-specific T cells in draining LNs, and the second is the long-term survival of the expanded T cell population. Functional TRAF2 protein expression appears to be important for both phases of the OX40-enhanced Ag-specific T cell response. OX40-mediated down-regulation of CTLA-4 most likely contributes to the enhanced early expansion of Ag-specific T cells, but not their survival.

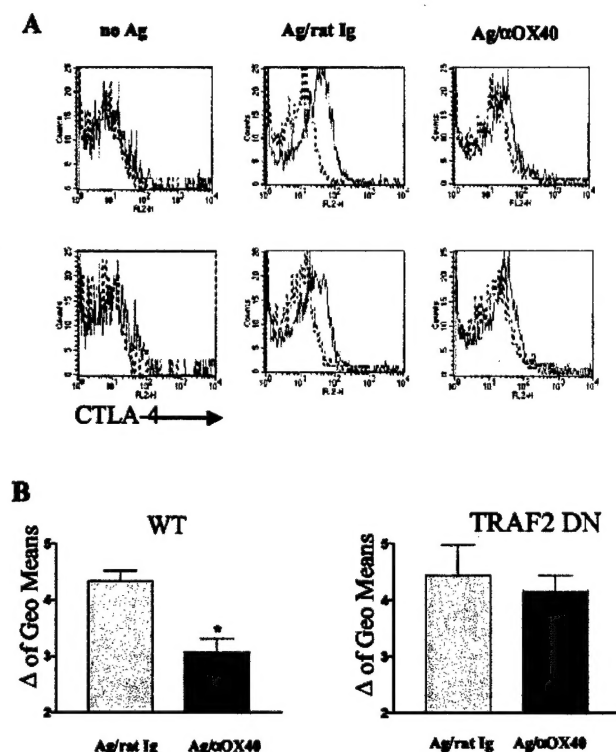


FIGURE 5. OX40-mediated decrease in CTLA-4 protein expression is TRAF2 dependent. Normal BALB/c mice received 1×10^6 TRAF2 DN⁺ KJ1-26⁺ (T2DN) or control (WT) KJ1-26⁺ T cells. Two days later, mice were primed with OVA and given either rat IgG or anti-OX40, as described in *Materials and Methods*. The mice were sacrificed 3 days after immunization, and the draining LN cells were prepared for Ab staining. The cells were triple stained for KJ1-26, CD4, and CTLA-4. Cells in each group were gated on KJ1-26⁺/CD4⁺ cells and then were assessed for CTLA-4 expression. **A** and **B**, Represent two independent experiments. The staining was performed on live cells to measure CTLA-4 that had appeared on the cell surface. **A**, The raw histograms for CTLA-4 expression of cells gated on KJ1-26⁺ and CD4⁺ are shown for WT T cells from unimmunized mice, mice immunized with Ag and anti-OX40, or Ag and rat Ig. In **B**, the graphs represent the change in the geometric means of the isotype control Ab compared with the CTLA-4 Ab. Error bars represent SE of three mice/group, and engagement of OX40 in WT T cells showed a significant decrease in CTLA-4 expression (*, $p = 0.01$) compared with the rat Ig control WT group. There was no significant decrease in CTLA-4 expression when OX40 was engaged in TRAF2 DN T cells compared with the rat Ig control group.

The data presented provide evidence that the OX40/TRAF2 intracellular pathway is involved with the generation of long-lived Ag-specific T cell memory *in vivo*. TRAF2 has been shown to be associated with the cytoplasmic tail of several other TNFR family members, including TNF, CD40, and 4-1BB, and is essential for the biologic response mediated through the c-Jun N-terminal kinase/stress-activated protein kinase pathways (25–27). Signal transduction through the TRAF2 molecule has also been shown to activate p38 mitogen-activated protein kinase and NF- κ B-associated pathways (28). All three intracellular signal transduction pathways described above are connected to separate nuclear transcription factors that are known to activate proinflammatory gene products (e.g., cytokines and antiapoptotic proteins) in T cells (27, 29). We found that the potent T cell-enhancing signal mediated through engagement of OX40 is interrupted by impaired TRAF2 protein function; therefore, the downstream events must be associated with one or a combination TRAF2-specific signaling pathways. Recently, we have performed a similar experiment, as de-

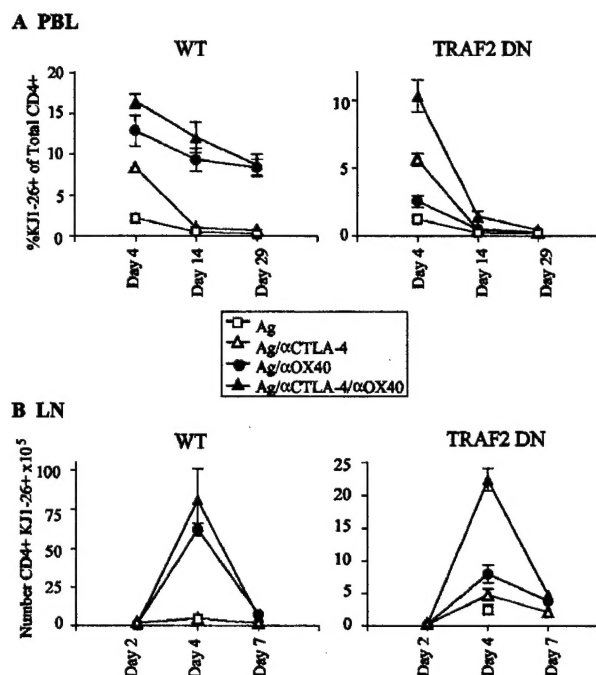


FIGURE 6. Anti-OX40 and anti-CTLA-4 coadministration partially restores the OX40-specific TRAF2 DN defect. Normal BALB/c mice received 1×10^6 TRAF2 DN⁺ KJ1-26⁺ (TRAF2 DN) or control (WT) KJ1-26⁺ T cells. Two days later, mice were primed with OVA and given either rat IgG, anti-OX40, anti-CTLA-4, or anti-OX40 and anti-CTLA-4, as described in *Materials and Methods*. **A**, The graphs depict a time course experiment in which blood was analyzed in the four groups shown on days 4, 14, and 29 after immunization for both WT and TRAF2 DN transfers. Lymphocytes were isolated, stained to detect the Ag-specific T cells, and analyzed by flow cytometry to obtain the percentage of Ag-specific T cells. The dose of anti-CTLA-4 given in this experiment was 100 μ g on days 0 and 1 administered s.c. The dose of anti-OX40 was the same as in the previous figures. Error bars represent SE of five mice/group. **B**, The graphs depict a time course experiment in which the draining LNs were analyzed in the four groups shown on days 2, 3, and 4 after immunization, for both WT and TRAF2 DN T cell transfers. Lymphocytes were isolated, counted, stained to detect the Ag-specific T cells, and analyzed by flow cytometry to assess the number of Ag-specific T cells per mouse. The dose of anti-CTLA-4 given in this experiment, 50 μ g on days 0 and 1 administered s.c., is suboptimal dose for enhancing Ag-specific T cell function in this assay (Evans and Weinberg, unpublished observation). The dose of anti-OX40 was the same as in the previous figures. Error bars represent SE of five mice/group. **A** and **B** are two independent experiments.

scribed in Fig. 2, with the TRAF1 knockout mice crossed onto the DO11.10 background. In contrast to Fig. 2, the TRAF1 knockout T cells showed no decrease or increase in OX40 augmentation; therefore, we feel the effects described in this manuscript are TRAF2 specific and not just a nonspecific TRAF-related phenomenon.

Although a substantial decrease in memory T cell generation was observed in the TRAF2 DN compared with WT T cells stimulated with anti-OX40, there was no difference observed in the TRAF2 DN and WT T cells stimulated with Ag alone *in vivo* (rat Ig groups). In the model system described in this work, we injected soluble Ag with no adjuvant. We have found that under these conditions the APC (dendritic cells or B cells) from draining LN express little, if any, OX40 ligand (Evans and Weinberg, unpublished observation). However, others have shown that injection of Ag in the presence of a potent adjuvant (CFA) induced high levels of OX40L on APC (30). Therefore, we speculate that the reason there was no difference observed in T cell responses between WT and TRAF2 DN rat Ig groups immunized with Ag is that the APC

do not express OX40L (or other TNF family member costimulators) and cannot deliver a signal to OX40⁺ T cells within the draining LNs. A recent article that supports this hypothesis describes the use of the same TRAF2 DN mice infected with influenza virus (31). Live virus is known to provide danger signals that can activate APC, and Cannons et al. showed that the TRAF2 DN mice were deficient in viral-specific CD4 T cell cytokine production 7 days after infection and secondary/memory CD8 T cell responses were impaired. The authors also show there was no defect in APC function in this mouse strain and speculated that all the immune defects were specific for T cell function. The viral data suggest that OX40 as well as other T cell-specific TNFRs (e.g., 4-1BB) known to be associated with TRAF2 function were most likely essential for normal physiologic antiviral T cell function.

We feel that a strength of the experimental design was the allowance for targeting OX40 directly via Ab stimulation in vivo within the TRAF2 DN T cells. A disadvantage of the model may be that the signal delivered through the OX40-specific Ab might not exactly mimic physiologic stimulation of T cells through OX40L⁺ APC. Several investigators have now shown that OX40 and OX40L knockout mice are deficient in memory T cell responses, mainly in the CD4 T cell arm (7, 32–34). Gramaglia et al. (7) quantified Ag-specific memory T cells responses via limiting dilution in the OX40-deficient mice 30 days after immunization with Ag in two separate adjuvants (CFA and alum/pertussis). With both adjuvants there was a 10- to 20-fold decrease in CD4⁺ memory T cell development in the OX40 knockout mice compared with WT mice. In our experiments, we found a 10- to 20-fold increase in CD4⁺ memory T cell development by injecting the OX40 Ab in vivo without adjuvant. Therefore, the endogenous signal generated through OX40L expressed on an APC or in vivo OX40 Ab signaling appears to produce similar outcomes as far as memory T cell survival. However, there are differences that occur between endogenous and Ab signaling through OX40, which include enhanced Ag-specific Ab production with OX40 Ab treatment. In the majority of OX40/OX40L knockout studies to date, there have been no differences observed in Ag-specific Ab production between immunized WT and OX40 knockout mice (35). Another difference appears to occur when mice are immunized in the presence of a strong adjuvant (CFA). Under these conditions, the OX40 ligand is expressed on APC, and anti-OX40 still enhances CD4⁺ T cell responses, although not to the extent observed when mice are immunized with soluble Ag (7). These data suggest that either the signal generated via OX40 Ab stimulation is a stronger signal than the endogenous OX40L stimulation or the Ab can target more OX40⁺ T cells than OX40L⁺ APC.

To gain a better understanding of the biologic pathways downstream of OX40 signaling that mediate enhanced T cell expansion and survival, we examined the genes that were up- or down-modulated during OX40 engagement in vivo by gene array analysis (Table II). There were several genes of interest that were down-regulated upon OX40 engagement, including Mad4, CD27, L-selectin, Fas, and CTLA-4. A 13-fold decrease in the expression of the transcriptional repressor Mad4 was observed. Mad4 is known to associate with the *c-myc* interacting protein Max, and the Mad/Max complex has been shown to suppress transcription initiated by *c-myc* (36–38). Therefore, the OX40-stimulated T cells should have increased *c-myc* activity, which others have shown is associated with increased cell cycle progression (proliferation) and an increase in cell size (38), both of which were consistent with what occurs upon OX40 engagement. Another consequence of *c-myc*-specific transcription is an increase in apoptotic cell death, which is the opposite of what has been observed in the OX40-stimulated T cells (38). Fas is associated with the induction of apoptosis, and

was down-regulated by T cells following OX40 engagement. This may account, at least in part, for the increased survival of OX40-stimulated T cells. The gene array data also showed an OX40-mediated decrease in CD62L and CD27 mRNA by these recently stimulated effector T cells, which could influence their migration (39). Most notably, the CD62L low and CD27 negative CD4⁺ T cells will home to nonlymphoid tissues, including the lung, skin, and gut (39). Previously, we showed that OX40 engagement can alter the migration of T cells, resulting in increased Ag-specific T cells migrating to the blood (23). Others have also shown an increase in the chemokine receptor CXCR5 found on T cells isolated from transgenic mice that overexpress the OX40L (40), although we observed no increase in CXCR5 in our analysis.

The mRNA for two cytokine receptors showed substantial changes upon OX40 engagement 3 days after immunization. mRNA for the IL-6R was decreased in the OX40-treated group, while the IL-2R α -chain was increased (Table II). Signaling through IL-6 may be detrimental to the development of memory T cells, although currently there are no data to support this hypothesis. Alternatively, the increase in IL-2R mRNA suggests that the OX40-stimulated T cells may be able to proliferate after Ag exposure for a longer duration of time, and therefore yield greater numbers of Ag-specific cells. Further evidence in support of this hypothesis is displayed in Table II, in which a 24.6-fold increase was observed in the DNA polymerase regulatory subunit for the OX40-stimulated T cells.

Because OX40 function is dramatically decreased in T cells expressing the TRAF2 DN mutant protein, it allowed us to focus on OX40-related gene products that were TRAF2 dependent. This was accomplished by comparing gene array analysis of WT and TRAF2 DN T cells isolated from hosts stimulated with Ag and anti-OX40. This analysis was performed on T cells isolated 3 days after immunization, and surprisingly, we found very few differences between the two groups. Two biologic replicate samples were performed and compared for genes that were altered in expression. Of the 12,000 genes screened, we found only 20 differences between the two groups. CTLA-4 and L-selectin (CD62L) were two gene products of known biologic function that were down-regulated upon OX40 engagement in a TRAF2-dependent manner. L-selectin is a protein associated with T cells migrating to LNs, and, as discussed above, down-regulation of mRNA might affect trafficking of the Ag-specific T cells. Because of the potent biologic activity associated with CTLA-4 and T cell function (24), we focused on this molecule as a potential TRAF2-dependent regulator of OX40 function.

To date, not much is known about the transcriptional control of CTLA-4 gene expression, but these data show that engagement of OX40 decreases mRNA levels at the critical day 3 time point, and this decrease appears to be TRAF2 dependent. To link the OX40-mediated decrease in CTLA-4 protein expression to biologic function, we attempted to reverse the OX40-specific TRAF2 DN defect by CTLA-4 blockade in vivo. Although blockade of CTLA-4 was able to overcome the early proliferation defect attributed to TRAF2 DN protein expression, the effect was short-lived and not able to accentuate the long-term survival of Ag-specific T cells (Fig. 6A). Therefore, we feel there are other molecules involved that are OX40/TRAF2 dependent and associated with long-term T cell survival. It is also apparent from the data that TRAF2-independent events are involved with the OX40-mediated stimulation of T cells, as illustrated in Fig. 4B. We hypothesize that engaging OX40 involves a complex assortment of signaling events and adapter proteins that work in concert to eventually provide the enhanced T cell function in vivo. However, TRAF2 appears to be

a critical adapter molecule that is required for both OX40-enhanced early T cell proliferation and the eventual conversion of those cells into long-lived memory T cells.

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